DNA vaccines - challenges in delivery

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Current Opinion in Molecular Therapeutics (2000) 2(2):188-198
 ISSN 1464-8431

DNA vaccines are typically comprised of plasmid DNA molecules that encode an antigen(s) derived from a pathogen or tumor cell. Following introduction into a vaccinee, cells take up the DNA, where expression and immune presentation of the encoded antigen(s) takes place. DNA can be introduced by viral or bacterial vectors or through uptake of 'naked' or complexed DNA.

Vaccination with DNA is a recent technology possessing distinct advantages over traditional vaccines (killed or attenuated pathogens) and the more recently developed subunit vaccines. Unlike most subunit vaccines, DNA vaccines induce both the humoral and cellular arms of the immune response. The stimulation of both arms of the immune system is important not only for the prevention of many diseases including AIDS, but also allows the use of a vaccine for therapeutic purposes. While the traditional attenuated pathogen vaccines are also able to elicit both cellular and humoral immune responses, there is a risk of reversion from the attenuated state to the virulent state. This risk does not exist with DNA vaccines. DNA vaccines can be manufactured and formulated by generic processes.

DNA vaccine technology, however, is still in its infancy and much research needs to be done to improve the efficiency with which these vaccines work in humans. While continued efforts toward improving both DNA expression and DNA delivery are equally important for increasing the utility of DNA vaccines, this review will focus both on non-viral delivery of plasmid DNA and delivery methods for the encoded antigen.

Keywords DNA condensation, DNA delivery, DNA vaccines, intracellular trafficking, lipoplex, transfection

Introduction

Unlike conventional drugs, plasmid DNA molecules are macromolecular and take part in complex interactions with cellular and extracellular biomolecules. Pharmacological activity of these prodrugs is dependent on their ability to be targeted to appropriate cell types, to be transported across the cell membrane, to have some degree of nuclease resistance and to maintain steady-state concentrations in the appropriate intracellular compartments. The polar and the anionic nature of plasmid molecules do not readily allow transfer across biological membranes [1]. Research into the delivery of nucleic acids has primarily focused on the delivery of antisense, triplex forming and ribozyme oligonucleotides, wherein the desired pharmacological properties have been developed into the structure without sacrificing the ability of oligonucleotide DNA and RNA molecules to function in base-pairing and catalysis. However, these developments cannot be readily translated to the delivery of plasmid DNA molecules, as they do not preserve the DNA molecule's ability to be expressed within the host cell. Therefore, the delivery of plasmid molecules has utilized either 'naked' DNA or agents that interact non-covalently with plasmid molecules.

In order for DNA vaccines to function, the cells of the vaccinee must internalize the DNA component of the vaccine. Although 'naked' DNA can be taken up by cells *in vivo*, the efficiency of the process is poor. Approximately 10^8 DNA molecules are taken up by approximately as many cells following the intramuscular inoculation of > 10^{10} molecules [2•]. Furthermore, the internalized DNA must then be transported to the cell nucleus such that the encoded antigen gene(s) can be transcribed and translated by the host cell enzymes. As both the processes of DNA internalization and nuclear localization are inefficient, the development of novel DNA delivery systems will be needed before DNA vaccines can realize their full potential. Structure-based rational designs for DNA delivery are limited. In addition, it is unclear as to which cell types should be targeted for DNA delivery for the optimal elicitation of immune responses. Therefore, current strategies are based both on rational design and empirical analysis [3]. It is the scope of this review to discuss current research and thinking in the field. Efforts to develop passive and active, and targeted and non-targeted plasmid DNA delivery systems will be described.

The route of DNA administration plays a fundamental role in DNA delivery. By altering the route of DNA administration, the magnitude and type of immune response can be modulated [4,5]. The modulation is thought to be effected through: (i) delivery of DNA to and subsequent transfection of different cell types; (ii) a difference in transfection efficiency at different sites; and/or (iii) differences in the transfected cell environment. A summary of the data available from these studies is presented.

Altering antigen delivery can also modulate immune response. There has been much innovation in the design of the DNA vaccine encoded antigen(s), with attempts to increase MHC class I and/or MHC class II presentation. Strategies have focused on both the intercellular and intracellular delivery of antigen to antigen-presenting cells (APCs) and to various antigen-processing pathways within a cell. Much has been learned from studies involving antigen delivery and these will be reviewed.

The success of DNA vaccines may require engineering of the immune response, possibly through the use of cytokines and costimulatory molecules. Inviting relevant cell types to participate in the uptake and processing of the antigen molecules is predicted to be relevant to the successful development of DNA vaccines. Literature in this area is reviewed with particular emphasis on conceptualization of the approaches and on the future of such approaches towards successful vaccine development.

DNA condensation

DNA condensation is a compaction process by which the inter-atomic distances are minimized between adjacent nucleotides. Condensation of DNA is a prerequisite to the uptake of DNA by cells, as condensed DNA occupies less space, there are more molecules per unit of space and there are fewer size-related restrictions on cell uptake. Although the major resistance to condensation is electrostatic repulsion, resistance is also contributed to by other unfavorable free energies. The relative contributions of these unfavorable free energies vary by several orders of magnitude [6,7••]. In supercoiled DNA (a topological state induced by two interwinding DNA strands where the number of interwindings is greater than the number of helical turns in the duplex) the free energy barriers are temporarily overcome for a given subset of sequences in a DNA molecule. The resultant torsional forces are transmitted into other parts of the molecule to relieve torsional stress, resulting in single stranded loops within the supercoiled molecule. Therefore, the supercoiled topological form is actually in equilibrium with other variant topological forms within a given DNA molecule.

The potent electrostatic barriers to DNA condensation can be overcome through the use of cations. Charge neutralization of DNA by cations relieves electrostatic repulsion, allowing DNA condensation to occur [8,9]. This free energy change causes a molecular collapse of plasmid DNA resulting in a 5- to 6-fold decrease in the hydrodynamic radii of the plasmid molecules, with a concomitant 2- to 3-orders of magnitude decrease in volume occupancy [10,11]. Condensation of DNA achieved through the use of cationic lipids, cationic microspheres, proteins and peptides will be discussed in the following sections. In addition to condensation, complexation with certain condensing agents confer other biophysical properties to the complexed DNA and to the surface architecture of the transfecting molecule.

Lipoplexes

Cationic lipids and their role in the complexation of DNA into lipoplex formation

Cationic lipids and cationic amphiphiles are ion-pair reagents that have both lipophilic and hydrophilic properties. Solubility of the lipid in aqueous solution is dependent upon the structure of specific hydrophilic and lipophilic groups. Depending upon the hydrophobic composition of the molecule, cationic lipids and amphiphiles can adopt a number of different liposomal structures. In polar solvents, a single chain lipid with a cationic headgroup will assemble into micelles containing hydrophobic cores, while certain lipids containing two or more hydrophobic chains can form bilayered vesicles [12]. A more complex liposomal structure is formed when a co-lipid or DNA is added. The multi-lipid interactions that occur through the addition of co-lipids bring about local rigidity in the otherwise fluid environment of mono-lipid liposomes, which further increases the asymmetry of the complex, causing a reassortment of bilayers (if present) and the possible creation of bilayers where originally there were none. Particles with distinctly different biophysical properties from the mono-lipid liposomes are thereby generated. Differences in these properties may impact the ability of these particles to be taken up by cells [13]. In addition, the presence of some neutral co-lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol appear to increase transfection capabilities through processes hypothesized to invoke endosomal evasion or escape following cellular uptake [14].

Addition of DNA to cationic liposomes results in the charge neutralization of both the cationic lipids and DNA. Both the charge neutralization of DNA and the reordering of DNA on the cationic headgroups result in condensation of the DNA molecule. A concomitant reordering of the lipids in the liposomes also occurs upon addition of DNA. Unless controlled assembly processes are used, the resultant lipoplex particles are heterogeneous in both size and structure.

Lipoplexes as a facilitator of in vitro transfection

Complexes of DNA and lipids made by adding DNA to preformed cationic liposomes (lipoplexes), have been found to greatly facilitate the transfection of DNA into tissue cultured cells. Transfection of cells by lipoplexes was originally thought to occur by fusion of the lipoplex with cellular membranes. In fact, the observed transfer of phosphatidyl lipids from lipoplexes into cellular membranes was taken as proof for lipoplex/cell membrane fusion. Several laboratories have shown, however, that liposomes do not generally fuse with cellular membranes, rather the uptake is through a passive phagocytic or endocytic process [15,16]. Entry of DNA appears to be facilitated through increased residence time near an actively phagocytic cell. It has also been suggested that the presence of lipoplexes in the vicinity of a cell may in fact induce phagocytosis and or endocytosis to occur in that cell.

Aggregation of lipoplex particles has been observed to occur during cell transfection procedures. Transfection of cells is induced by, or occurs with, particles of a certain size range only; other sized particles are refractory to or actually inhibit this process. In order to increase the efficiency of DNA delivery to cells, it will be important to further elucidate the biochemical and biophysical properties of the transfecting particles. It will also be necessary to develop controlled assembly processes, such as those described by Gregoriadis and Szoka [17,18], in order to reproducibly generate lipoplex particles that are homogeneous in both size and structure.

Lipoplexes and the immune response

DNA immunization using non-viral vectors requires efficient methods for the delivery of plasmid DNA to cells *in vivo*. Although lipoplexes made by adding plasmid DNA to preformed cationic liposomes have been successful in

enhancing transfection in cell culture, these cationic lipid-DNA complexes, in general, do not result in increased transfection after *in vivo* intramuscular injection. In fact, these complexes appear to inhibit both the expression of the complexed DNA and the generation of subsequent immune responses when compared to the use of DNA alone [19•,20].

Dehydrated-rehydrated vesicles (DRVs) [17,21••], comprised of phosphatidylcholine (PC), DOPE, cationic lipids and DNA, enhance immune responses relative to those responses elicited by DNA alone when administered intramuscularly [21••] or subcutaneously [22]. Although these vesicles can be of a similar chemical composition to the traditionally made lipoplexes, DRV particles are smaller and more uniform in size. In addition, recent data suggests that the basic architecture of DRVs is different from that of traditionally made lipoplexes. Using anion competition, DNA was displaced from the traditionally formed lipoplexes but not from DRVs, suggesting that the DNA in lipoplexes is externally bound but is internalized in DRVs [23]. DNA may be similarly competed from lipoplexes by negatively charged proteins *in vivo*, perhaps explaining the failure of these complexes as DNA delivery vehicles for DNA vaccination. Although protection of DNA from nucleases has previously been cited as a proof of DNA entrapment within traditionally made lipoplexes [24], the premise of these experiments is questionable since DNA complexed with cationic lipids may not be a nuclease substrate. The true substrate is DNA coordinated to a divalent cation through the oxygen atom of the phosphoryl group.

As DRVs (but not traditionally formed lipoplexes) function in the intramuscular and subcutaneous delivery of DNA, it seems likely that the biophysical properties as well as the chemical composition of cationic lipid DNA complexes must be taken into consideration for the development of DNA delivery vehicles.

Local anesthetics and their role in DNA vaccine technology

Although naked DNA can transfect muscle cells *in vivo*, pretreatment of muscle with the local anesthetic bupivacaine several days prior to injection of DNA, results in increased DNA uptake, as evidenced by increased DNA expression at the injection site [25,26]. Increased expression of DNA is also associated with increased immune responses to antigens encoded by the injected DNA [27]. Increase in muscle transfection by pretreatment with bupivacaine is thought to be due to the myogenic activity of bupivacaine. Treatment of muscle with bupivacaine causes muscle fiber degeneration and subsequent recruitment of myoblasts into muscle regeneration [25]. Dividing myoblasts are postulated to be more likely to be transfected with DNA than the non-dividing, differentiated muscle cells, and are presumably responsible for the observed increase in muscle cell transfection. In addition, the recruitment of inflammatory cells to the site of bupivacaine injection may also allow for transfection of immune cells [25,26,28,29]. Due to the temporal nature of muscle cell degeneration and regeneration, the window of opportunity to transfect regenerating muscle fibers is 1 to 7 days post-bupivacaine treatment.

It has been shown that mixtures of DNA and bupivacaine delivered simultaneously also result in increased transfection and expression in muscle cells. The mechanism of muscle cell transfection observed for simultaneous delivery of DNA and bupivacaine is not related to the mechanisms involved with bupivacaine pretreatment [Pachuk CJ, Ciccarelli RB, Samuel M, Bayer M, Troutman R, Zurawski D, Schauer JI, Sosnoski DM, Higgins TJ, Weiner DB, Zurawski V Jr, Satishchandran C, manuscript in preparation]. Interactions between bupivacaine and DNA have also been discovered that may explain the observed increase of *in vivo* transfection when bupivacaine and DNA are administered simultaneously.

Microspheres and cochleates

Microspheres are biodegradable particles designed for slow drug release. Adsorbed or entrapped DNA has been successfully delivered, following intramuscular injection, to APCs using microsphere particles comprised of PLG (poly(lactide-co-glycolide)) [30-32]. Efficiency of uptake by APCs is dependent upon microsphere size. As microspheres afford considerable protection to entrapped DNA, they are potential DNA delivery vehicles for mucosal and oral routes of immunization. In fact, oral DNA vaccination with microspheres has been shown to induce both mucosal and systemic immune responses to the encoded HIV envelope antigen [33].

Microspheres coated with cationic molecules to which the DNA is then adsorbed, allow for higher DNA expression following *in vivo* cellular uptake when compared to 'naked' DNA [34]. Intramuscular administration of these cationic microparticles also increases the immune response to the encoded antigen by 3-orders of magnitude, relative to the 'naked' DNA control [34].

Cochleates are 'jelly-roll' like structures formed by the bridging of neutral lipids in liposomes [35]. This bridging is mediated by divalent cations such as Ca^{2+}. Cochleates are stable in biological fluids and have been successfully used to deliver protein antigens.

Plasmid DNA can also be entrapped into cochleates and delivered to cells *in vivo* [36]. Due to the exclusion of water during cochleate formation, entrapped plasmid DNA molecules are predicted to be stable to acid environments, and hence these particles may be of use for oral DNA vaccine administration. Future research will determine the viability of this approach for the delivery of plasmid-based vaccines.

Targeted and active transfection processes for *in vivo* DNA delivery

Cellular uptake and nuclear localization of DNA follow the rules of mass action. Maximum bioavailability of DNA can only be realized through active transfection processes, which can be achieved through either physical means or through the use of targeted DNA delivery systems.

Physical methods of DNA delivery include biolistic injection, electroporation and iontophoresis. Although these processes afford rapid uptake of injected DNA, transfection

of DNA is limited to the surfaces and areas that are accessible to the devices themselves. In some studies, gene gun technology, which utilizes biolistic injection, has been found to elicit immune responses of much higher magnitude than those elicited through the use of DNA alone [37].

Targeted DNA delivery systems are biochemical approaches involving the use of ligands that interact with and bind to cell surfaces. These ligands can be complexed with DNA through cationic molecules [38,39] or through oligonucleotides that form triplex strands with DNA. DNA can be targeted to specific cell types through the use of a ligand or an antibody specific for that cell type [40,41•,42]. Alternatively, DNA can be actively transfected into any cell type by targeting receptors present on all cell types.

Conferring targeting capabilities to the DNA is predicted to result in only modest increases in *in vivo* delivery. Following the initial cell surface interactions, the DNA delivery vehicle should be designed to be able to either fuse with the cell membrane or to participate in an active cellular uptake mechanism. Use of efficient cell attachment and entry mechanisms derived from viral systems, such as Sendai virus, have resulted in 10-fold more efficient liposome uptake compared to those liposomes lacking this additional viral feature [43,44].

A problem associated with ligand-mediated DNA delivery systems, is that the internalized complex becomes localized within the endosomal compartment. However, the presence of membrane disrupting agents within the complex can allow escape from the endosome [38,39]. Internalized DNA must also be translocated to the nucleus where it can be expressed. Current research has focused on the use of nuclear localization signals that can be linked to the DNA through the use of either peptide nucleic acids (PNAs) that form triplex structures with the plasmid DNA molecule or modified lysines [45,46•].

Although targeted delivery systems are the most desired methods for DNA delivery, the methods to achieve them are still unclear. Knowledge of the desired cell types to target and which cell types to avoid are primary requirements for the successful development of these systems. As our knowledge continues to grow, it is not unrealistic to conclude that non-viral gene delivery systems will reach the efficiency and specificity of viral-based delivery systems.

Routes of administration

In order to modulate the level and type of immune response, plasmid DNA has been administered to human and animal vaccinees through a number of different routes and by a number of different methods. Until the actual mechanisms of immune stimulation effected by DNA vaccines are determined, the optimal site and/or method of administration may need to be determined on a case-by-case basis. For example, different types of immune response eliminate various pathogenic microorganisms. Knowledge gained regarding the precise means by which immune effectors are stimulated following each method and route of DNA administration will allow researchers and clinicians to direct particular DNA vaccines in a way which stimulates the immune response in a particular manner.

Intramuscular Immunization

Intramuscular (im) injections are often used to administer DNA vaccines, frequently leading to a robust immune response. Uptake of plasmid and expression by myocytes in the area of injection has been demonstrated [47••]. It is doubtful, however, that myocytes are acting as APCs due to low expression of essential immune costimulatory molecules, such as B7-2 [48]. The unlikely possibility of myocytes acting as APCs has been eliminated by studies using MHC-chimeric mice in which bone marrow-derived cells were shown to be the actual APCs responsible for immunity following DNA vaccine administration [49,50••,51]. Dendritic cells (or possibly macrophages [52]) have been proposed as the primary APCs following im injection [53], although it is not yet clear if these cells were directly transfected with plasmid DNA or had taken up protein produced by transfected myocytes (a phenomenon known as crosspriming [54•]). One study involving a DNA vaccine encoding non-secreted protein has suggested, however, that direct expression of protein inside APCs may be necessary for the generation of immunity following im injection [55].

The stimulation of antibody production following DNA vaccination may be occurring through similar mechanisms. Antibodies are usually generated following the ingestion of foreign (exogenously produced) protein by APCs, which then digests these proteins to peptides for subsequent presentation to the immune system. This would indicate that cells transfected with plasmid DNA must express the protein and transfer it to classical APCs. However, non-secreted proteins have been shown to induce some levels of antibody production following DNA immunization [56]. These proteins may perhaps be released from the cell by apoptosis or may be secreted via a non-classical pathway.

Cutaneous Immunization

The presence of a large number of resident APCs (Langerhans and dendritic cells) in the skin makes this area an advantageous site of immunization. Intradermal (id) immunization with DNA vaccines has proven to be an effective method of generating a specific immune response. In fact, topical application of DNA to the skin leads to a measurable immune response to the encoded antigen [57]. Coating of plasmid DNA onto gold beads and delivering them into cells with a 'gene gun' was more effective in eliciting an immune response when compared to DNA given im, intranasally (in) or intravenously (iv) [58]. A number of studies have shown that cells at the site of immunization are necessary for the induction of the immune response; both migratory and non-migratory cells are probably important [59-62].

It has been observed, in general, that gene gun-immunization typically leads to a Th2 response, while delivery of plasmid via needle injection induces a Th1 response [63]. Exceptions to this 'rule' have been noted [64] and the type of response generated may be, at least partially, antigen-dependent. The larger quantities of DNA needed for im injection may non-specifically push the immune response in a Th1 direction because of immune stimulatory sequences (ISS) present on plasmid DNA [65]. A Th1 response can be elicited following gene gun immunization if plasmids containing the genes for *interleukin-12* or *interferon-α* are codelivered [66].

Mucosal immunization

An effective induction of mucosal immunity would be advantageous in preventing disease, as mucosal sites are the points of entry into the body for a variety of pathogenic bacteria and viruses. Selective enhancement of secretory IgA (sIgA) and specific cytotoxic T-lymphocytes (CTLs) in regional lymph nodes has been attempted through in, oral and intravaginal delivery of DNA vaccines. To prevent degradation of the DNA, it has been necessary to form complexes between the DNA and various (usually lipidic) substances. Expression of the appropriate protein has been observed in lungs [58], nasal tissue [67], spleen and draining lymph nodes [68] following in administration of DNA vaccines. Intranasal administration of plasmid containing the genes for HIV *env* and *rev* lead to increases in specific CTLs in the spleen and regional lymph nodes [69]. While in administration of DNA alone usually results in a Th2 response [69], this can be skewed in the Th1 direction by complexing with various lipidic substances [68,70] or by co-administration of plasmids containing the genes for the appropriate cytokines [69].

The goal of an oral DNA vaccine is complicated by the need to protect the DNA during its journey through the stomach. Microencapsulation of plasmid DNA into particles containing PLG lead to both specific mucosal IgA and serum antibodies [30]. Intestinal IgA and specific serum antibodies for rotavirus VP6 antigen, as well as some degree of protection from challenge, were observed following oral administration of PLG-encapsulated plasmids containing the *VP6* gene [71]. Oral delivery of plasmids in other formulations have also led to specific mucosal immune responses [72].

Specific antibodies (IgG and IgA) were found in vaginal washes following intravaginal administration of plasmids containing the HIV *env* and *rev* genes [73]. Similar results were found when rats were immunized intravaginally with the gene for human growth hormone [74]. Intravaginal immunization of a pregnant chimpanzee with genes from HIV led to specific IgA and IgG in the saliva and sera but not in vaginal washes [75].

While a great deal of research continues in the area of mucosal DNA immunization, it is clear that much remains to be learned and that administration of DNA through multiple sites may be necessary to achieve protective immunity.

Presentation of antigen

Intercellular and intracellular targeting of antigen can be accomplished through simple changes in antigen design. Rerouting of antigens via these changes modulates both the level and the type of response, presumably by increasing MHC class I and or MHC class II presentation.

MHC class I

The intracellular production of antigen following the administration of DNA vaccines is the hallmark of DNA vaccination. Intracellular antigen synthesis allows MHC class I presentation of antigen-derived peptides that can prime cellular immune responses. The generation of peptides for MHC class I presentation is, in part, mediated through the ubiquitination of endogenously produced proteins. Ubiquitination occurs when the C-terminal glycine of ubiquitin forms an amide isopeptide linkage with the ε-amino group of one or more exposed lysine residues of a substrate protein. Once linked to the substrate protein, ubiquitin itself becomes a target for ubiquitination, resulting in the polyubiquitination of the substrate protein. The polyubiquitinated substrate protein is then degraded by the 26S proteosome complex. A subset of the degradation products is comprised of peptides that can associate with MHC class I molecules.

The magnitude of a cellular response may be heightened by increasing the efficiency with which an antigen is targeted for ubiquitination. Our knowledge of ubiquitination signals is limited however, and there appear to be multiple signals. In addition, ubiquitination is catalyzed by a myriad of ubiquitin carrier proteins that are specific for certain proteins [76•]. As the expression of these carrier proteins is also regulated according to cell type and cell cycle, it seems unlikely that there is a universal system for ubiquitination of all proteins in all cell types, at all points in the cell cycle. Nonetheless, there have been some reported successes at targeting DNA vaccine-encoded antigens into the ubiquitin pathway. Rodriguez *et al* have demonstrated enhanced CTL induction in mice immunized with DNA constructs encoding an N-terminal ubiquitin fusion protein [77,78]. Enhanced responses were seen when ubiquitin was fused to either a viral gene or minigene. The ubiquitin moiety of these fusion proteins is thought to be a substrate for ubiquitination, thereby targeting the entire fusion protein to the proteosome complex. This would require that a lysine residue become exposed in the ubiquitin moiety. As folding of the ubiquitin moiety is expected to be modulated by the downstream fusion partner, exposure of a lysine residue is not predicted to occur in all fusion proteins. The universality of this strategy will undoubtedly be tested over the next few years.

Another strategy for attempting to increase cellular immune responses has involved the removal of signal peptides from antigens destined for secretion. This forces the cytosolic localization and the increased intracellular accumulation of antigen proteins, which may allow for increased MHC class I presentation of antigen peptides. Initial data has shown that mice immunized with plasmids expressing cytosolic localized HSV-2 gD antigen have higher lymphoproliferative responses to gD than mice immunized with plasmids expressing secreted or membrane-associated gD [Higgins TJ, Herold KM, Arnold RL, McElhiney SP, Pachuk C, unpublished data]. Boyle *et al* have demonstrated higher CTL responses in mice immunized id with a DNA vaccine expressing cytosolic localized antigen [79]. Interestingly, when immunized im, higher CTL responses were seen in those mice receiving constructs expressing secreted or membrane-associated antigen. It is not clear why this apparent discrepancy exists.

In addition to accessing the MHC class I processing pathway by an endogenous route, virus-like particles (VLPs) and some proteins appear to also access this pathway exogenously [80,81]. Following secretion or export from cells, these VLPs

and proteins gain entry to the cytosol of other cells (perhaps by binding to a cell surface receptor), where they gain access to the MHC class I processing pathway. Therefore, for a subset of antigens, cytosolic localization and or proteosome targeting may not be necessary for the elicitation of optimal cellular immune responses.

MHC class II

The intracellular production of antigen that occurs following DNA vaccination, results in a population of antigen molecules that become localized within APCs. It is unclear whether the antigen found in APCs merely reflects antigen taken up by an APC or whether some antigen is actually synthesized in an APC; most likely it is combination of these two events. Localization of antigen within an APC allows for the MHC class II presentation of antigenic peptides. The production of these peptides is achieved by antigen degradation in a lysosome-dependent pathway.

There has been much innovation in the design of DNA vaccines in an attempt to enable more efficient class II presentation of antigen. These modifications have included alterations to the design of the antigen molecule, expression of accessory molecules and changes in regulatory elements such as the promoter.

In general, changes to the antigen are designed to result in the increased availability of antigen to APCs. These changes most often involve modifications that allow the antigen to be exported out of the cell. Comparisons of immune responses following DNA vaccination with plasmids expressing secreted or cell-associated antigen, have demonstrated that it is possible to increase the magnitude of the immune response by targeting the antigen for secretion. For example, both Geissler and Inchauspe have reported higher seroconversion rates and higher antibody titers in BALB/c mice immunized with plasmid constructs expressing various forms of secreted hepatitis C (HCV) core protein, as compared with those mice immunized with constructs expressing cell-associated core [82,83]. Secretion of bovine herpesvirus 1 gD (84) and HSV-2 gD has also been shown to induce higher antibody titers in mice relative to those mice receiving constructs encoding cell-associated forms of the antigen [Higgins TJ, Herold KM, Arnold RL, McElhiney SP, Pachuk C, unpublished data]. In addition, the serum immunoglobulin isotype profile was different in mice immunized with constructs expressing secreted gD; expression of cell-associated gD was associated with a predominance of serum IgG2a, while higher serum levels of IgG1 were associated with expression of the secreted form of the antigens. While secretion of a variety of other antigens has also enhanced antibody responses [85], there are examples where no significant differences in immune response were seen in animals immunized with constructs expressing secreted versus cell-associated forms of antigen [86]. Perhaps the conflicting results can be explained by differences in serum level antigen concentrations resulting from differences in antigen expression or antigen stability.

As an alternative to secreting antigen from a cell via the use of secretory signals, the co-expression of proteins or peptides that induce cellular apoptosis may have merit. Not all proteins can be secreted from a cell, due to the presence of domains that interact with intracellular membranes. In these instances, induced apoptosis following antigen synthesis would allow spillage of antigen from the cell, resulting in increased antigen availability to APCs. In addition, cells undergoing apoptosis may recruit inflammatory cells to the site, and thereby increase antigen delivery to APCs. Consistent with this, a self-replicating RNA vaccine was recently demonstrated to be more efficacious than a traditional plasmid-based vaccine. The increased efficacy was postulated to be due to apoptosis of cells harboring self-replicating RNA.

It has recently been demonstrated that secreted antigen can be targeted directly to APCs by a further modification of the antigen. Boyle *et al* have made C-terminal antigen fusions to CTLA4 [87••]. CTLA4, a ligand present on activated T-cells, binds to B7 molecules expressed on the surface of APCs. The presence of CTLA4 on the antigen fusion protein should allow targeting of the antigen to B7-expressing cells, thereby increasing the efficiency with which antigen is taken up by APCs. Experimental data suggests that this was in fact achieved. Mice immunized with plasmids expressing the B7 targeted antigen had much higher antibody levels than those mice immunized with the non-targeted antigen; at 2 weeks post-immunization, mice receiving the CTLA4 fusion protein plasmid had antigen-specific antibody titers 10,000-fold higher than mice immunized with the native antigen plasmid. In addition, a 7000-fold increase in IgG1 levels was seen in mice immunized with the CTLA4 fusion construct as compared to mice immunized with the plasmid expressing the non-targeted antigen.

In addition to antigen modifications that allow targeting of antigen to specific cell types, modifications have also been incorporated which allow intracellular targeting of antigen. Expression of antigen as a fusion protein to lysosome-associated membrane protein (LAMP-1) has been shown to result in endosomal/lysosomal localization of antigen. LAMP-1 contains a localization signal that re-routes antigen into the lysosomal compartment and therefore into the MHC class II processing pathway. Immunization with constructs expressing LAMP-1-antigen fusion proteins has been shown to result in enhanced CD4+ presentation of antigen. In addition, immunization of mice with a vector expressing a LAMP-1/tumor antigen fusion protein was found to protect mice from tumor challenge, whereas mice vaccinated with a vector expressing the unmodified tumor antigen were not protected [88]. However, the use of LAMP-1 fusion proteins does not always result in increased immune response and more research must be done in this area before the utility of this approach can be assessed [89].

Other modifications can be made at the level of promoter choice. The use of dendritic cell- and macrophage-specific promoters would allow for the production of antigen in these APCs following uptake of plasmid DNA into these cells. It will be interesting to see if expression of antigen within APCs results in enhanced immune responses.

Molecular adjuvants and modulators of immune response

Of the different ways to modulate the immune response to DNA immunization, one of the most promising may be through the co-administration of 'biological' adjuvants such

as cytokines. Cytokines are molecules secreted mainly by bone marrow-derived cells that act in an autocrine or paracrine manner to induce a specific response in cells expressing the appropriate cytokine receptor. Other adjuvants include the costimulatory molecules that help signal T-cell activation and expansion. The large number of studies identifies this area of DNA vaccine research as one of the most reproducible and important areas for further development.

Codelivery of IL-2, IFNγ or IL-4 expression cassettes in BALB/c mice

Of the different ways to modulate the immune response to DNA immunization, one of the most promising may be through the co-administration of 'biological' adjuvants such as cytokines. Multiple laboratories have reported that co-injection of plasmids encoding cytokines can have a substantial effect on the immune response to plasmid-encoded antigen, for example, in multiple viral and cancer antigen systems [90-93].

Two important cytokines that have been examined for their immune modulatory activities are granulocyte-macrophage colony-stimulating factor (GM-CSF) a putative Th0 cytokine, as well as the prototypic Th1-inducing cytokine IL-12. IL-12 plays a critical role in the Th1 immune response mainly by inducing production of the Th1-associated cytokine IFNγ. In contrast, GM-CSF is a hematopoietic growth factor, which stimulates neutrophil, monocyte/macrophage and eosinophil colony formation. It also induces proliferation and differentiation of erythroid and megakaryocyte progenitor cells. GM-CSF also increases the antibody-dependent cell-mediated cytotoxicity of neutrophils, eosinophils and macrophages but has not been reported to have a direct role in the generation of CTL response *in vivo*.

The effect of co-expressing the genes for murine *IL-12* and *GM-CSF* with DNA vaccine cassettes for HIV-1 has been studied [94,95]. CTL responses were increased up to 5-fold using the IL-12 plasmids but little effect was observed on CTL induction by GM-CSF using equivalent doses of plasmids. Lower doses were frequently less impressive for cellular immune modulation. Humoral responses were also studied. In repeated experiments IL-12 suppressed specific antibody responses by 10 to 20%, while GM-CSF appeared to have the opposite effect, increasing antibody responses 4- to 6-fold. The boost in serological responses with GM-CSF correlated with increased neutralizing antibody responses. This humoral effect is likely the consequence of increased CD4+ Th activity specific for B-cell responses.

Activation and proliferation of Th-cells play a critical role in inducing both a humoral immune response via expansion of antigen-activated B-cells and a cellular immune response via expansion of CD8+ cytotoxic T-lymphocytes. In further adjuvant experiments, a dramatic increase in proliferation (4-fold) was observed in animals co-immunized with antigen plasmids and IL-12 plasmids. Interestingly, a similar and sometimes even more dramatic increase in the stimulation index was observed in animals co-immunized with antigen plasmid and GM-CSF plasmid. It was interesting that both treatments appeared to increase effective helper T-cell responses yet they clearly had different effects on the induction and maintenance of an antigen-specific CTL response. Recently, similar studies have been performed in a large primate chimpanzee model. In these studies increased proliferation was also observed with *GM-CSF* as well as *IL-12* gene co-immunization.

The activity of GM-CSF and IL-12 was compared with other Th1-type cytokines for their ability to enhance *in vivo* immunity. In these studies, GM-CSF, IL-12 and IL-2 all enhanced the T-cell proliferative response [96••,97••]. It was somewhat surprising that IL-2 and IL-15 had significantly different activities in this model. However, it suggests that a DNA vaccination approach can give important insight into the subtle differences associated with these similar but non-overlapping vaccine adjuvants *in vivo*. This became more evident as CTL responses were evaluated. In general, in several studies IL-12 was the best driver of MHC-restricted CD8+ CTL activity, however, IL-15 also demonstrated significant potency. Interestingly, IL-18, which can drive IFNγ induction in a similar manner to IL-12, was a poor adjuvant for CTL responses.

Studies have been performed to determine the effects of such adjuvants on survival in an animal challenge system. These studies used a herpes simplex virus (HSV) model system. The HSV system has the advantages of lethal mucosal challenge as well as titrateable challenge doses. The model grants insight into the general value of this approach. Surprisingly, all of the Th1 cytokines increased vaccine potency. These included IL-12, IL-2, IL-15 and IL-18. In comparison with Th2 cytokines the immune responses induced were entirely different and the survival profile was similarly different. Th2 cytokines delivered as vaccine adjuvants actually decreased survival supporting a direct effect on the memory response. In these studies there were discernable differences between the different test groups. IL-12 was always the best mediator of protection and survival from lethal challenge. Additionally, IL-12 as well as the aforementioned GM-CSF controlled pathogenesis as evidenced by the almost complete lack of herpes-induced lesions in surviving mice.

In conclusion, evaluation of the adjuvant effects of cytokine co-immunization suggests that while IL-12 may be the best candidate to date for enhancing the cellular immune responses to plasmid antigens encoded by DNA vaccine cassettes, IL-15 (through expanding CTL responses) and GM-CSF (through expanding T-helper responses) also significantly enhanced antigen-specific cellular immune responses. These results indicate that further combination studies examining the cytokine approach are warranted.

Codelivery of B7-1 or B7-2 expression cassettes in BALB/c mice

Although antigen is expressed at significant levels in muscle following im inoculation of plasmid DNA, it is well established that muscle cells do not express the costimulatory molecules required for efficient antigen presentation. Among the different costimulatory molecules, B7-1 (CD80) and B7-2 (CD86) have been investigated by a number of groups as possible vaccine adjuvants. The B7-1 and B7-2 molecules interact with the CD28/CTLA4 molecules on T-cells providing an important second signal

in addition to ligation of the T-cell receptor through the MHC peptide complex [98]. These two signals then facilitate expression of IL-2 receptor and progression of the T-cell through the cycle of immune activation. B7-1 and B7-2 are upregulated during the antigen presentation process, most likely following CD40/CD40 ligand interactions between T-cells and APCs.

The genes for *B7-1* and *B7-2* have been tested as vaccine adjuvants in a similar manner to the cytokine studies outlined above. In these studies dramatically greater levels of proliferation were observed in the mice splenocytes collected from animals immunized with antigen plasmids and pCD86 (a 6-fold increase). In contrast, CD80 exhibited more modest effects on T-cell proliferation than CD86. Examination of CTL responses revealed that the animals immunized with antigen plasmids alone or antigen pulse plus pCD80 showed low levels of CTL responses. In contrast, co-immunization with pCD86 resulted in a dramatic increase in CTL activity that also resulted in an increase in CTL precursor frequency.

These results are consistent with the hypothesis that codelivery of a costimulatory molecule with DNA dramatically improves immune responses by providing more efficient antigen presentation. It has recently been reported that APCs that take up DNA increase their expression of costimulatory molecules [52].

Codelivery of CD40 or CD40L expression cassettes in BALB/c mice

Costimulatory pathways are important avenues for gleaning new and functional DNA vaccine adjuvants. As B7-2 adjuvant activity likely involves subsequent induction of the CD40L, the effects of this pathway on immune induction have been investigated. The ability of CD40L, as well as CD40, to enhance the humoral and cellular immune responses to plasmid antigen vaccines has also been researched [Sin JI, Weiner DB, manuscript in preparation]. Co-immunizing mice with either B7-2 (CD86) or CD40L plus antigen expression cassettes led to enhanced antigen-specific T-cell proliferation and CTL activity. B7-1 and CD40 enhanced cellular immune responses to a much lesser extent. These results identify specific costimulatory pathways, particularly B7-2 and CD40L as important vaccine adjuvants for further studies. It is not unlikely that changing the specific antigen could have dramatic effects on vaccine-induced immune responses and should be considered in further investigation of this approach.

Conclusions

In conclusion, the potential of plasmid DNA-based vaccines has sparked enormous activity in DNA delivery research. It is recognized that one of the major limitations to the success of DNA vaccines is its delivery. Condensed and entrapped DNA designed for active transfection methods are predicted to yield consistent and desired levels of transfection. Advances in controlled self assembly of DNA complexing agents, novel carriers for oral, skin and lung administration are expected in the near future. Intradermal inoculations have shown promise in several animal species for certain antigens. However, the rules that govern why certain antigens and certain sites perform differently from others is unclear. Further research is expected to clarify these issues as well as to develop a clear understanding of the cell types involved. Unraveling the intracellular pathways that need to be invoked to assure robust immune responses, and developments in engineering of the immune response through chemokines, costimulatory molecules and antigen delivery should also allow rational design of delivery vehicles for plasmid DNA vaccines.

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